Expression and Cellular Localization of Classic NADPH Oxidase Subunits in the Spontaneously Hypertensive Rat Kidney

Tinatin Chabrashvili, Akahiro Tojo, Maristela Lika Onozato, Chagriya Kitiyakara, Mark T. Quinn, Toshiro Fujita, William J. Welch, Christopher S. Wilcox

Abstract—Phagocytes generate superoxide anion ($O_2^-$) by a classic, 5-component NADPH oxidase. $O_2^-$ contributes to hypertension in spontaneously hypertensive rats (SHR). Therefore, we tested the hypothesis that NADPH oxidase expression is enhanced in the SHR kidney. We also analyzed the localization of NADPH oxidase components in SHR kidney. Renal NADPH oxidase was quantified by reverse transcription–polymerase chain reaction and Western blotting and was localized in SHR and Wistar Kyoto rat (WKY) kidney by immunohistochemistry. The mRNA for 5 subunits of phagocyte NADPH oxidase, and also for MOX1 and RENOX (NOX4), was detected in adult rat kidney. Kidneys of adult (10 weeks old) SHR had a significantly ($P<0.01$) greater mRNA for p47phox (SHR 0.81±0.05 versus WKY 0.37±0.01, arbitrary unit), which was confirmed by Western blotting (SHR 0.58±0.04 versus WKY 0.42±0.04, arbitrary unit; $P<0.05$) and by immunohistochemistry. This higher p47phox protein expression was also detected in young prehypertensive SHR (SHR 0.61±0.05 versus WKY 0.39±0.04, arbitrary unit; $P<0.01$). The 10-week-old SHR contained more modest but significantly ($P<0.05$) greater protein for p67phox (SHR 0.54±0.02 versus WKY 0.46±0.02). Immunostaining localized p47phox, p67phox, and p22phox in vasculature, macula densa, distal convoluted tubule, cortical collecting duct, and outer and inner medullary collecting ducts. The kidney of SHR expresses genes for all the main components of phagocyte NADPH oxidase, RENOX, and MOX1. There is a prominent increase in the SHR kidney of the mRNA, and protein expression of p47phox in the vasculature, macula densa, and distal nephron, which precedes development of hypertension. (Hypertension. 2002;39:269-274.)

Key Words: oxygen ■ oxidative stress ■ nitric oxide ■ macula densa

Aerobic organisms generate reactive oxygen species (ROS) during respiration and as a byproduct of specific oxidases. Principal among ROS are superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical (OH·). The balance between ROS production and the antioxidant defense system determines the degree of oxidative stress. Recent studies implicate the redox state as a determinant of many cellular decisions. Overproduction of ROS contributes to the pathophysiology of hypertension, endothelial dysfunction, renal and vascular injury, diabetes, and atherosclerosis.

Cytosolic enzyme systems contributing to oxidative stress include the extended family of NADPH oxidases. The robust oxidative burst of stimulated phagocytes utilizes the complete NADPH oxidase pathway. Mohazzab et al and Rajagopalan et al have identified NADPH oxidase as a major site of $O_2^-$ generation in intact arteries. Components of phagocyte NADPH oxidase have been identified in several tissue culture cells, including cultured human mesangial cells, vascular smooth muscle cells (VSMCs), endothelial cells, glomerular epithelial podocytes, kidney proximal tubular epithelial cells, and fibroblasts.

The phagocyte NADPH oxidase is a multimolecular enzyme. This is composed of a membrane-associated 22-kDa $alpha$-subunit (p22phox) and a 91-kDa $beta$-subunit (gp91phox), with cytosolic components composed of p47phox, p67phox, and p40phox. Assembly of these units also incorporates a small ATPase, Rac1/Rac2. In rat VSMCs and colon, gp91phox is replaced by the 56% homologous MOX1; in mouse proximal tubules, by the 57% homologous RENOX. The site of NADPH oxidase expression within the kidney should give insight into locations for ROS generation. Previously, we found that $O_2^-$ contributes to hypertension, renal vasoconstriction, and enhanced tubuloglomerular feedback response in spontaneously hypertensive rat (SHR) kidney, but the source of $O_2^-$ is not known. Therefore, we tested the hypothesis that NADPH oxidase is overexpressed in the SHR kidney.
Sites of Immunoreactive NADPH Oxidase Subunit Expression in the Kidney of 10-Week-Old SHR

<table>
<thead>
<tr>
<th>Nephron Site</th>
<th>p47phox</th>
<th>p67phox</th>
<th>p22phox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerulus</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Podocyte</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mesangial cell</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Endothelial cell</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Proximal tubule</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DL</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TAL</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MD</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DCT</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CCD</td>
<td>+</td>
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<tr>
<td>OMCD</td>
<td>+/-</td>
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</tr>
<tr>
<td>IMCD</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Vascullature</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Interstitial fibroblast</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

DL indicates thin descending limb of loop of Henle; TAL, thick ascending limb; MD, macula densa; DCT, distal convoluted tubule; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; and IMCD, inner medullary collecting duct. Immunostaining expression: +, present; –, absent; +/-, weak.

In this report, we contrast the expression of the mRNA and protein for the components of phagocyte NADPH oxidase in adult SHR and Wistar Kyoto rat (WKY) kidneys. Because we detected higher p47phox expression in SHR, we determined if this increase preceded the development of hypertension. Finally, we located the immunocytochemical sites of expression of NADPH oxidase in the SHR kidney.

Methods

Animal Preparation
Experiments were performed on groups (n=6) of adult male SHR, age 10 weeks (250 to 280 g), and age-matched male WKY (Harlan Sprague-Dawley, Madison, Wis). The time course of changes in protein expression in the kidney of p47phox were undertaken on groups of 4-week-old prehypertensive SHR and WKY (50 to 70 g). The study was approved by the Georgetown University Animal Care and Usage Committee and was performed according to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 93-23, revised 1985) and Guidelines of the Animal Welfare Act.

Isolation of Total RNA
Total RNA was isolated from the kidney cortex using the guanidinium isothiocyanate method (Qiagen). DNase treatment was applied to avoid the contamination from genomic DNA.

Reverse Transcription and Quantitative Polymerase Chain Reaction
Quantitative multiplex reverse transcription–polymerase chain reaction (RT-PCR) was used to quantify the expression of mRNA for gene products for p22phox, gp91phox, p67phox, p47phox, p40phox, MOX-1, and RENOX as described previously. Quantitative multiplex PCR was done using synthetic oligonucleotide primers based on published sequences (see Table in online supplement).

Sequencing of PCR Products
Direct sequencing of reamplified PCR products was performed according to the DyePrimer and Dye Terminator system (Applied Biosystems) and analyzed on an Applied Biosystems Model 373A DNA sequencer.

Western Blotting
Western Blotting was performed as described previously. Signals were detected with previously characterized monoclonal19,20 and polyclonal antibodies20,21.

Light Microscopic Immunohistochemistry
Kidney slices were prepared and processed as described previously. Polyclonal antibodies20,21 directed against p22phox at a dilution of 1:200 or against p47phox and p67phox at a dilution of 1:400 for 2 hours were used to detect the signal.

Statistical Analysis
All values shown are mean±SE. Unpaired comparisons using Student’s t test were used to determine significance between specific groups. P<0.05 was considered statistically significant.

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

Results
RT-PCR reactions using oligonucleotide primers designed from previously described sequences for p22phox, gp91phox, p47phox, p67phox, p40phox, RENOX, and MOX1 yielded products of anticipated size (Table online). The GenBank contained no other similar sequences. No PCR products were obtained in samples not subjected to RT, indicating the absence of genomic DNA contamination. Direct DNA sequence revealed identical sequences for the rat kidney partial cDNA sequences for p22phox and MOX1 and for the published rat aortic smooth muscle cell sequences (GenBank Nos. U18729 and AF152963, respectively). Comparison of the sequences for p22phox, gp91phox, p47phox, p67phox, p40phox, and RENOX to mouse and human counterparts revealed high homology (Table online).

Using quantitative multiplex RT-PCR, we found the adult SHR kidney cortex from 10-week-old rats had a significantly (0.81±0.05, P<0.01) greater mRNA abundance for p47phox compared with that of the adult WKY (0.37±0.01) (Figure 1). There were no significant differences in expression of p22phox, p91phox, p67phox, p40phox, MOX1, or RENOX between 10-week-old SHR and WKY kidney cortexes (data not shown).

The immunoreactive expression of p22phox, p47phox, and p67phox proteins in rat renal cortex homogenates using polyclonal antibodies were confirmed for each of these proteins using monoclonal antibodies. There was a specific
band for each subunit at a molecular weight corresponding to that predicted for NADPH oxidase subunits in human and mouse proteins. Figure 2 shows the reaction of monoclonal antibodies to p47phox, p67phox, and p22phox with the rat kidney cortex homogenates, and the positive control with leukocyte lysate from rat blood. Western blot analysis in 10-week-old rats showed that p47phox was greater in SHR kidney (SHR 0.58 ± 0.02 versus WKY 0.42 ± 0.02, P < 0.05), as was p67phox (SHR 0.54 ± 0.02 versus WKY 0.46 ± 0.02, P < 0.05) (Figure 2). There was no detectable difference for p22phox (SHR 0.51 ± 0.02 versus WKY 0.49 ± 0.02, P < 0.05). The higher p47phox protein expression in the kidney of 10-week-old SHR compared with age-matched WKY was also detected in prehypertensive 4-week-old SHR, (SHR 0.61 ± 0.05 versus WKY 0.039 ± 0.04, P < 0.01) (Figure 3).

Immunocytochemical staining for all of the components studied was stronger in SHR than WKY kidney. Therefore, data shown is for SHR kidney, although a generally similar pattern was seen in WKY kidneys. Light microscopic observation of 2-μm wax sections from 10-week-old SHR kidney demonstrated that p22phox, p47phox, and p67phox were expressed strongly in the wall of the renal artery, whereas the expression was prominent in the smooth muscle cells (Figure 4). All of these components where also expressed strongly in the apical regions of the macula densa (MD) cells, cortical thick ascending limbs (TAL), distal convoluted tubules, and cortical collecting ducts (Figure 4). Higher magnification studies of the glomerulus showed that p47phox was expressed in podocytes (Figure 4). A prominent expression of p47phox protein was also detected in the MD and distal convoluted tubule segments of young prehypertensive SHR (4-week-old) (Figure 5). The immunostaining for 3 subunits of NADPH oxidase along the nephron segments of 10-week-old SHR is summarized in the Table.

**Discussion**

The primary new findings are that the mRNA corresponding to the 5 subunits of the classic phagocyte NADPH oxidase in addition to the mRNA for MOX1 and RENOX and the proteins for p22phox, p47phox, and p67phox is expressed in the normal adult rat kidney. The expression of the proteins for the subunits of NADPH oxidase occurs at specific nephron sites. Relative quantitative RT-PCR showed the mRNA expression of p47phox was 2.1-fold higher in kidney cortex of 10-week-old SHR compared with WKY. Western blot analysis detected an increase in p47phox and p67phox protein in SHR kidney cortex. The p47phox protein expression was higher in the kidney cortex of young 4-week-old SHR.

The rat kidney partial cDNA corresponding to p22phox, gp91phox, p47phox, p67phox, and p40phox was closely homologous with that cloned from human and mouse phagocytes. These represent renal genes, because the kidneys were perfused extensively with PBS to flush out blood compo-
nents. Moreover, immunocytochemical studies detected no neutrophils and only very occasional tissue macrophages in the kidney sections examined.

Although we detected mRNA for gp91phox, MOX1, and RENOX in the kidney, we were not able to address the issue of the protein expression for gp91phox and its homologues because antibody specificity could not be assured.

Proteins corresponding to p22phox, p47phox, and p67phox were located in the nephron of the SHR at the MD segment, TAL, apical areas of the distal convoluted tubule, and the cortical and medullary collecting ducts. The proximal nephron was not stained. The constitutive expression of these components in the SHR kidney, which was prominent at the luminal membrane, indicates that phagocyte-type NADPH oxidase may generate O$_2^-$ at these nephron sites in the SHR kidney. The TAL, the MD, and the cortical collecting ducts all express NO synthase constitutively.$^{22}$ NO generated in tubular epithelium and MD cells can regulate NaCl trans-

port.$^{23,24}$ Therefore, the finding of co-expression of NADPH oxidase components with NOS at specific sites in the nephron suggests that it could regulate the effects of epithelial-generated NO.

Previously, Bachmann et al.$^{25}$ reported the expression of mRNA for p22phox in the peritubular fibroblasts of the rat renal cortex. This was confirmed by immunocytochemical expression of p22phox protein in fibroblasts. Cultured podocytes from human glomeruli expresses the mRNA and immunoreactive proteins for p22phox, gp91phox, p47phox, and p67phox.$^{12}$ In the present study in the SHR, we detected the expression of only p47phox in podocytes.

The finding that the SHR kidney has an increased abundance of mRNA for p47phox and of protein for the p47phox, and p67phox subunits suggests that NADPH oxidase may be a source of the excessive renal production of O$_2^-$, which has been demonstrated in previous physiological studies.$^{4,15}$ The SHR has enhanced excretion of 8-iso-PGF$_{2\alpha}$, which is a
marker of oxidative stress. Moreover, long-term administration of tempol, which is a membrane-permeable $\text{O}_2^-$ dismutase mimetic, reduces the blood pressure and the oxidative stress of the SHR.\textsuperscript{15}

What are the possible functional correlates of this study? First, the expression of a complete NADPH oxidase system along the luminal membrane of the MD suggests that $\text{O}_2^-$ generated at this site may form a barrier, which limits locally generated NO from reaching targets on the luminal membrane. NO inhibits Na\textsuperscript{+} reabsorption at the TAL,\textsuperscript{23,26} MD,\textsuperscript{27} cortical collecting ducts,\textsuperscript{28} and the inner medullary collecting duct.\textsuperscript{29} Consequently, activation of an NADPH oxidase at these sites could impair the bioavailability of NO, which is implicated in the regulation of distal nephron Na\textsuperscript{+} reabsorption and activation of MD cells. The SHR kidney has an enhanced tubuloglomerular feedback response, which has been associated with a diminished buffering by MD-derived NO, despite a greater mRNA and protein abundance for type I NO synthase expressed in MD.\textsuperscript{30} This defective NO response in the SHR is restored by local microperfusion of MD cells.\textsuperscript{31} This suggests that local generation of $\text{O}_2^-$ in the TAL, MD, and the inner medullary collecting duct may act to protect the cotransporter from inactivation by NO, thereby contributing to enhanced NaCl reabsorption. Second, the finding that the renal cortical expression of p47phox is enhanced as early as 4 weeks of age suggests that it could be important in hypertension. At 4 weeks of age, the blood pressure of SHR is no higher than that of age-matched WKY.\textsuperscript{32} Therefore, it appears that the enhanced p47phox expression in the kidney precedes the development of hypertension in this model. Other studies have shown that the degree of oxidative stress in the blood vessel wall increases with age of the SHR.\textsuperscript{33} Because tempol can normalize the hypertension and renal vasoconstriction in adult SHR,\textsuperscript{4} it is possible that an enhanced activity of NADPH oxidase within the kidney may predispose to the development of hypertension in the SHR model.

**Acknowledgments**

The work was supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (DK-36079 and DK-49870) and National Heart, Lung and Blood Institute (HL-66575 and HL-68686) and by funds from the George E Schreiner Chair of Nephrology, Chagriya Kitiyakara was supported by fellowship training grants from the International Society of Nephrology and the American Society of Nephrology, National Capital Affiliate.

**References**


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Hypertension. 2002;39:269-274
doi: 10.1161/hy0202.103264

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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